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Synthesis of Multiple Peptides on Plastic Pins

Scanning protein sequences by bioassay for smaller bioactive peptide sequences requires a source of many peptides homologous with the parent protein sequence. This unit deals with one of the synthetic methods for making such sets of peptides (see Fig. 9.7.1). The key to preparing large numbers (hundreds to thousands) of synthetic peptides in a short time and at minimal cost is to use a parallel synthesis technique which is efficient and can be done on a small scale. The multipin technology is suitable because it can be performed without expensive synthesizers and it uses equipment available to most laboratories. Prior experience with organic synthesis techniques or peptide chemistry is useful but not essential. The products of synthesis by multipin technology are unpurified peptides which are useful as screening reagents and may also be used to prepare purified peptide on a small scale.

Most multipin techniques exploit the conventional 8 × 12 matrix layout of common microtiter equipment which simplifies handling of the synthesis, the products (peptides), and the test results. Computer assistance with synthesis and data analysis also speeds the cycle from designing the experiment through analyzing the results.

With multipin technology, peptides are synthesized in parallel on plastic "pins" (Fig. 9.7.2) to give sets of peptides suitable not only for B and T cell epitope scanning but also for other bioassays. Peptides can be either permanently bound to the surface of the plastic for direct binding assays, or they can be released into solution. There is a choice of N- and C-terminal peptide endings. For solution-phase peptides, the synthesis scale can be 1 or 5 μmol (for a 10-mer, ~1 mg or 5 mg, respectively). The preferred coupling/deprotection chemistry used is the milder 9-fluorenylmethyloxycarbonyl (Fmoc) protection scheme rather than the older *t*-butyloxycarbonyl (*t*-Boc) protection scheme (see *UNIT 9.1*), thus reducing the level of chemical safety risk arising from synthetic peptide chemistry.

This unit covers the strategy of the multiple peptide approach to biological scanning, the synthetic protocols, and the handling of peptides after synthesis—cleavage, preliminary purification, storage, and analysis (see Basic Protocol). It is specific for the multipin technique using equipment obtained from Chiron Technologies, although some of the approaches are applicable to other multiple synthesis techniques. Procedures for multipin equipment obtained from other suppliers may differ from the procedures described here, and the manufacturer's literature should be consulted. This unit also includes protocols for preparing Fmoc-amino acid solutions (see Support Protocol 1) and for acetylating (see Support Protocol 2) or biotinylating (see Support Protocol 3) synthesized peptides.

STRATEGIC PLANNING

For a protein whose primary structure is known, the conceptually simplest method of locating all the bioactive linear peptide sequences is to make all possible peptide subsets of the protein sequence and test them. If only selected parts of the sequence are synthesized, or only the predicted active parts, bioactive sequences could be missed. The use of a set of highly overlapping peptides likewise reduces the possibility that the most bioactive sequences might be missed because they are absent from the set. For example, a set of all overlapping 20-mers offset along the sequence by one residue at a time should capture the entire set of helper T cell epitopes, and this is a much more reliable approach than trying to predict motifs. In reality, a synthetic peptide scan through a protein is a compromise between the cost and effort in making and screening all peptides and the need for completeness. Thus, one worker may choose to make all overlapping 8-mers to

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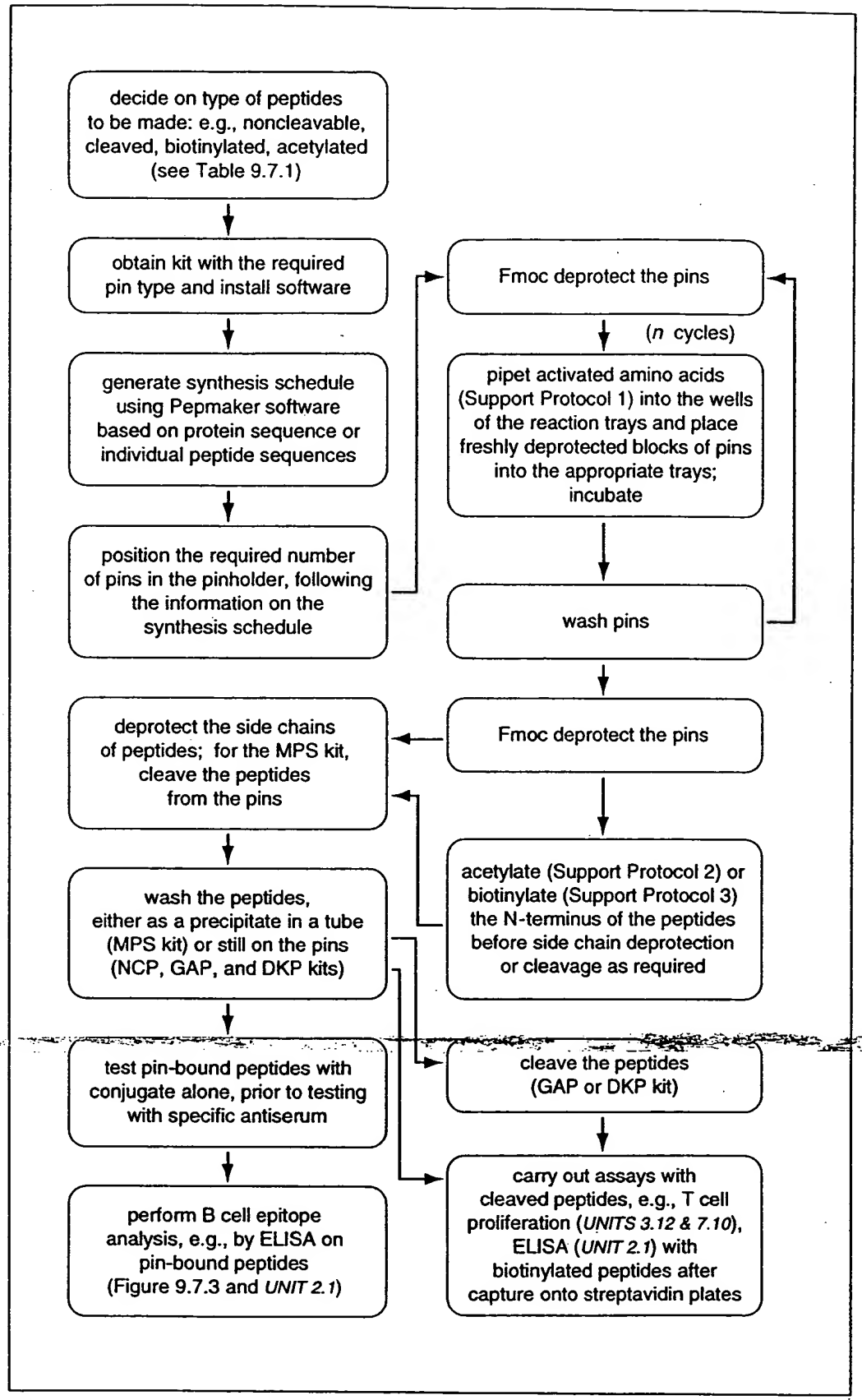

Figure 9.7.1 Flow chart for multipin peptide synthesis.

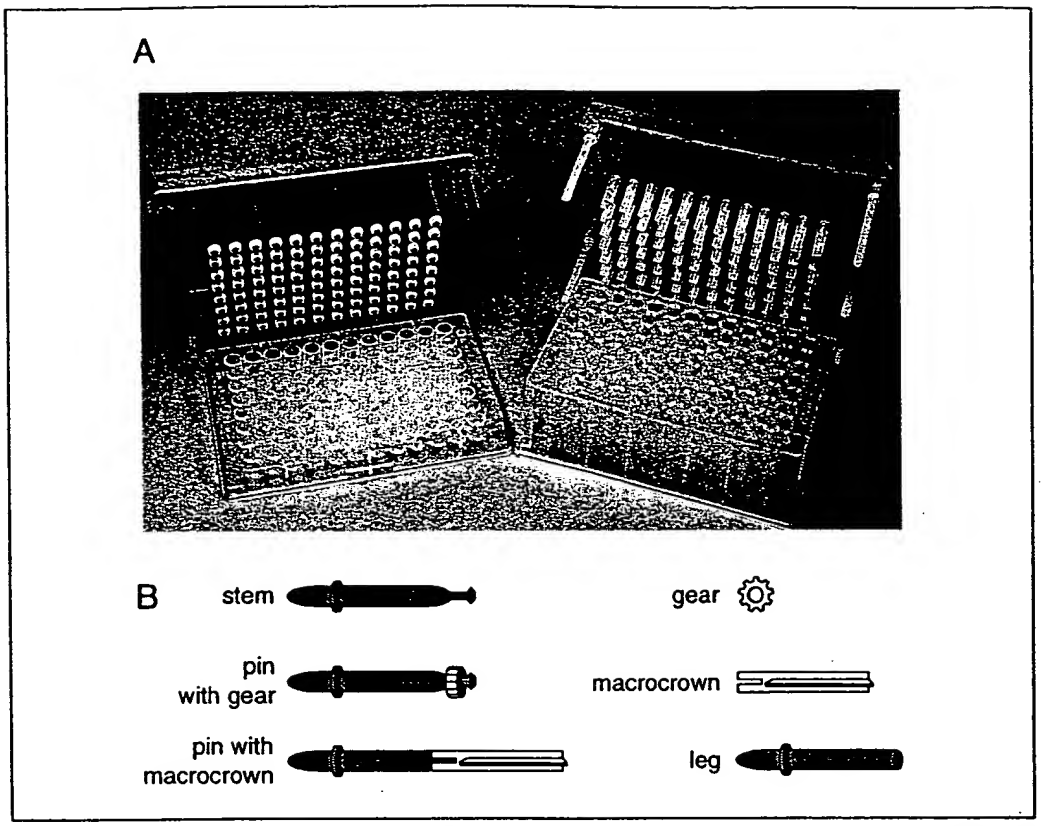

Figure 9.7.2 Apparatus for multipin peptide synthesis. (**A**) Assembled synthesis block with 96 gears (left) or 96 macrocrowns (right). (**B**) Components of the pin assembly. Components are either push-fitted together (e.g., legs or stems into the pin holder) or clipped on (gears or macrocrowns onto stems). All components are solvent-resistant plastic, either polyethylene, polypropylene, or copolymers of these two monomer types.

find the linear (continuous) B cell epitopes, and another may make 12-mers offset along the sequence by five residues for the same purpose. In each case, all sequences of eight residues from the protein are present in at least one peptide, but the latter approach requires only one-fifth the number of peptides.

Planning the Synthesis

Synthetic peptides are assembled by solid-phase synthesis one amino acid at a time, commencing with the C-terminal end of the peptide on the solid phase (see *UNIT 9.1*).

The assembly process, or coupling, requires activation of the α-carboxyl group of each incoming amino acid to make it reactive with the α-amino group of the growing peptide chain. To prevent unwanted polymerization or side reaction, reactive groups in each amino acid must be temporarily protected, and the protecting group removed before further reaction can be carried out. The protecting group on the α-amino function of the most recently added amino acid must be removed before another amino acid can be coupled to it, so the α-amino protection must be labile under conditions that do not remove side-chain protection. Later, the side-chain-protecting groups must be removable under conditions that do not attack the peptide bonds. The two common protecting group "schemes" are known as *t*-butoxycarbonyl (*t*-Boc) or 9-fluorenylmethyloxycarbonyl (Fmoc). The protecting group scheme currently recommended for multipin peptide synthesis is the milder Fmoc scheme, which is the only scheme described in this chapter.

Table 9.7.1 Types of Pins for Multipin Peptide Synthesis[a]

Name	Linker[b]	Physical format[c]	Loading	Final form of peptide
NCP	Noncleavable	Gear	50 nmol	(N-capping)-PEPTIDE-linker-pin
MPS	AA ester	Macrocrown	5 μmol	(N-capping)-PEPTIDE-acid
MPS	Rink amide	Macrocrown	5 μmol	(N-capping)-PEPTIDE-amide
DKP	DKP-forming	Gear	1 μmol	(N-capping)-PEPTIDE-DKP
GAP	Glycine ester	Gear	1 μmol	(N-capping)-PEPTIDE-glycine-acid

aAbbreviations: DKP, diketopiperazine; GAP, glycine acid peptide; MPS, multiple peptide synthesis; NCP, noncleavable peptide; (N-capping), a free amine, acetyl group, or biotin; PEPTIDE, the sequence of the peptide being made.

bNature of linker between peptide and graft polymer on the pin: noncleavable linker, β-alanine-hexamethylenediamine; DKP, diketopiperazine; AA ester, amino acid ester; Rink amide, Rink amide–forming linker.

cSee Figure 9.7.2B.

Before beginning to plan the actual synthesis in detail, a choice needs to be made regarding how the peptides will eventually be presented in the bioassay. The options available to investigators are listed in Table 9.7.1.

For noncleavable peptide (NCP) kits, peptides are permanently bound on the solid phase (pin surface) and can be used for direct binding assays but not for interaction with living cells or other complex (e.g., multicomponent) systems. In this case, the peptides must be "regenerated" between repeat assays by disrupting the peptide-ligand interaction without damaging the peptide. The quantity of peptide made is very small (50 nmol), but it is sufficient to provide a high surface density of peptide for direct binding assays.

In the other options, peptides are synthesized on pins and then released into solution. The mechanism of peptide release into solution affects the postsynthesis handling and thus the suitability of peptides produced by each cleavage method for various assay systems.

For multiple peptide synthesis (MPS) kits, the released peptides have a "native" free acid or an amide carboxy terminus. To make free acid C-termini, it is necessary to use macrocrowns that already have the first (C-terminal) amino acid on them because the chemistry of forming the first (ester) link is too difficult for the inexperienced user. In contrast, the Rink amide linker allows formation of a peptide with a C-terminal amide of any amino acid by adding the C-terminal amino acid to the Rink handle macrocrown using the standard amino acid coupling protocol. A Rink amide linker is a linker that can accept an amino acid but then can be cleaved in trifluoroacetic acid (TFA) to release the amide form of that amino acid (Rink, 1987). Although acid or amine endings are often the most desirable peptide format to have, they are also the most complex to produce because the cleavage of the peptides from the pin is into neat TFA plus scavengers which needs to be evaporated to recover the peptide. The scale of peptide synthesis for MPS kits is 5 μmol (~5 mg of a decamer).

For glycine acid peptide (GAP) kits, peptides with a glycine at the carboxy terminus are cleaved as the free acid, so that the C-terminal residue is a natural amino acid (glycine) and is not blocked. The peptides are also relatively simple to release from the pin and require little postsynthesis handling. However, the presence of glycine at the C-terminus may be undesirable where the C-terminus plays an important role in peptide bioactivity. The scale of synthesis for GAP kits is 1 μmol (~1 mg of a decamer).

In diketopiperazine (DKP) kits, peptides are synthesized with a DKP group at the C terminus. The DKP group is a cyclic dipeptide formed from C-terminal lysine and proline residues during the facile cleavage of the peptide under the mildest possible conditions:

neutral aqueous buffer. In applications where the presence of the DKP group is acceptable, this type of peptide can make the downstream processing of synthetic peptides very simple and fast. The peptides can be placed into a bioassay system immediately after completing the cleavage. The scale of synthesis for DKP kits is 1 μmol (~1 mg of a decamer).

For these five kit options, it is also possible to choose a variety of N-terminal endings on the peptides. For example, it may be desirable to acetylate pin-bound peptides (see Support Protocol 2) to eliminate the positive charge that would otherwise be present on the α-amino group of the N-terminal residue, or to enhance the activity of a peptide in a T helper assay (Mutch et al., 1991). A handy option for cleaved peptides is to place a biotin group on the N-terminus (see Support Protocol 3) so the peptide can be captured using avidin or streptavidin. These additions must be made prior to side-chain deprotection of the peptides.

There are other configurations for multiple peptide synthesis—e.g., the SPOTS or "peptides on paper" system (Zenica/CRB), the RaMPS system (DuPont), and multi-synthesizer machines (e.g., Advanced ChemTech).

Assessing Peptide Sequences

Peptides differ so much in properties that it is important to assess the likely properties of the peptides before attempting to synthesize them. Peptide length and hydrophobicity are the two main attributes affecting successful synthesis. The longer the peptide, the lower will be the purity of the product, as each amino acid coupling cycle is never 100% efficient. Synthesis of peptides longer than 20 residues should be avoided unless special attention can be given to each sequence. Hydrophobic peptides may be difficult to synthesize, but more significantly they may be poorly soluble in aqueous buffers, restricting their ultimate usefulness in bioassays. Prior to beginning synthesis of a set of peptides, it is sensible to assess them all for hydrophobicity (Fauchere and Pliska, 1983; *UNIT 9.3*) and decide if all should be attempted as they stand. In many cases, it is possible to choose slightly different peptides (longer, shorter, or using a different starting and finishing point in the homologous protein sequence) that will have more user-friendly properties.

As well as these general factors affecting peptides, particular peptide sequences may have characteristics that make them difficult to synthesize, or they may be problematic after synthesis. It is not feasible to discuss all the common problems here. To help assessment of peptide sequences, a software application called Pinsoft is available free from Chiron Technologies. This allows any sequence to be typed in and an assessment is automatically reported.

Generating Peptide Sequences

Computer software (Pepmaker) supplied with synthesis kits allows sets of overlapping peptide sequences to be generated from a protein sequence computer file using the single-letter amino acid code. Alternatively, sequences can be created using a word processor and the resulting computer text file can then be used by Pepmaker to guide synthesis. The use of this software simplifies the otherwise complex and tedious task of adding the right amino acids to each reaction plate on each synthesis cycle.

DO NOT expose pins to acetic anhydride at any other time except during acetylation. Also, do not store acetic anhydride anywhere near where peptide synthesis is performed.

The DMF does not need to be amine-free.

20% piperidine/DMF

Prepare a 20% (v/v) solution of the best quality piperidine available in analytical reagent–grade dimethylformamide (DMF). Prepare a fresh solution for each synthesis (solution can be reused several times within a synthesis). Store at room temperature in an amber bottle containing activated molecular sieves to remove moisture.

CAUTION: This solution is highly flammable and toxic.

If high-quality piperidine is not available, it may have to be treated with solid sodium hydroxide and redistilled.

DMF need not be amine-free.

Side chain deprotecting (SCD) solution

33 parts (v/v) trifluoroacetic acid
1 part (v/v) ethanedithiol
2 parts (v/v) anisole
2 parts (v/v) thioanisole
2 parts (v/v) H_2O
Prepare immediately before use and do not store or reuse

CAUTION: This solution is corrosive and extremely malodorous. Contamination of the laboratory, especially with ethanedithiol, should be avoided. Wipe the outside of ethanedithiol-contaminated equipment or containers with dilute, 0.1% aqueous hydrogen peroxide to oxidize ethanedithiol to a nonodorous compound before removing the container from the fume hood. DO NOT allow hydrogen peroxide to contact other readily oxidizable materials or reagents.

Sonication buffer

1% (w/v) SDS
0.1 M sodium phosphate buffer, pH 7.2
0.1% (v/v) 2-mercaptoethanol (2-ME)
Store at room temperature up to 1 week

CAUTION: Before discarding sonication buffer, destroy remaining 2-ME by adding 2 ml 30% hydrogen peroxide per liter of buffer.

COMMENTARY

Background Information

The multipin method was developed by Dr. H.M. Geysen and coworkers (Geysen et al., 1984, 1987) as a scanning method for linear antibody-defined epitopes. Eventually in the late 1980s, the method was adapted to parallel synthesis of cleaved (soluble) peptides (Maeji et al., 1990), opening the way for systematic scanning of T helper (Reece et al., 1993) and cytotoxic epitopes (Burrows et al., 1994). Initially only suitable for synthesis of short peptides (up to 10 amino acid residues), the method can now routinely produce peptides of up to 20 residues of acceptable quality for initial screening experiments (Valerio et al., 1993).

Critical Parameters

Successful peptide synthesis requires reagents of a quality appropriate to the particular step, and the careful application of those reagents. For example, the protected amino acids need to be free of reactive counterions such as dicyclohexylamine (DCHA), contaminating unprotected amino acid, isomers such as the D-amino acid, and water. Check carefully that the amino acid as supplied is EXACTLY the same as specified in the manual or on the software. Apart from quality testing each amino acid, the best assurance of quality is to buy only from reputable suppliers.

Dimethylformamide (DMF) is the primary solvent for carrying out reactions (couplings) on pins. Its low volatility and moderate polarity make it suitable for dissolving the amino acids and solvating the graft polymer/growing peptide on the pin surface. Purity is not critical for some (washing) steps, but is critical for the DMF used just before and during amino acid coupling. Presence of excessive amine in the DMF results in loss of activated amino acid because the amino acid couples to the amine rather than to the peptide on the pin. Fortunately, the pin system allows use of substantial molar excesses of incoming amino acid (typically 6- to 1000-fold), so loss of some amino acid is not disastrous. Fresh DMF of the best available grade should be used for the coupling, and it is recommended that the amine level be tested using the FDNB test (Stewart and Young, 1984).

Liberal use is made of methanol as a washing solvent. Analytical reagent grade methanol is readily available at low cost in large containers (20 or 200 liters) and is relatively easy to dispose of. It is possible to reduce the use of methanol by reusing it for washes: the last wash bath in any series should be in fresh (pure) methanol. In the next round of washes, the former last bath is then reassigned as the second-to-last wash, the previously second-to-last bath becomes the third-to-last, and so on. For each synthesis cycle, the first wash bath in the series is the one which is discarded. The presence of methanol is undesirable during reactions on the pins, but as it evaporates readily it can be easily removed by standing the block in a moving stream of air, such as the opening of an operating chemical fume hood. Methanol will dry more rapidly and the methanol-washed pins will take up less moisture from the air if the methanol is warm (e.g., prewarmed to 45°C in a closed bottle in a water bath).

Other solvents (e.g., ether, petroleum ether, acetonitrile) should be the best available grade.

Carrying out the correct synthesis of the peptides requires that all steps are performed with a very high level of attention to detail. All cyclically repeated steps (washes and deprotections) must be performed, and the activation and dispensing of the amino acids for each coupling cycle must be carried out exactly, or the peptides made may have the incorrect sequence, may be missing an amino acid, or may be truncated. Computerized equipment is available for assisting with the accurate dispensing of amino acids to the wells in a reaction tray (e.g., "Pin-Aid," Chiron Technologies; Carter et al., 1992). The growing peptides must not be subjected to conditions that would prematurely block or deprotect the side chains (for example, from premature exposure to acetic anhydride or trifluoroacetic acid which should be stored well away from where peptide synthesis is being performed).

As a spot test for correct completion of all the steps of synthesis, it is wise to synthesize controls on each block of 96 pins. For noncleavable peptides, these controls can be peptide sequences that can be probed with an antibody known to react with the peptide. In this case, one of the two peptides should be a negative control, such as a randomized sequence. For cleavable peptides, the quantity and quality of the controls can be monitored by the usual techniques of HPLC (*UNIT 9.2*), amino acid analysis, and mass spectrometry. Ultimately, proof that an assay result is a function of the particular peptide made has to rely on a confirmatory experiment carried out with more highly-characterized peptide or on analysis of a sample of the particular peptide used in the experiment.

Once peptides have been made, they need to be handled and stored carefully to prevent degradation. Noncleavable peptides (pins) should be stored dry in a refrigerator after removal of any bound protein. If stored with desiccant they should be stable for months to years. Cleaved peptides can be stored frozen or as dry powder. After a long period of storage, it is wise to reassay controls or confirm the quality of the stored peptide by analysis.

Another parameter critical to data from large numbers of peptides is to ensure that the identity of each peptide is properly tracked and that activity is not ascribed to the wrong peptide. Consistent use of the 8 × 12 microtiter plate format for synthesis, storage, assay, and use of computerized records for tracking all three processes can help avoid mistakes. Tracking and control is particularly easy if the assay data is read directly from a microtiter plate reader to a computer that is programmed with the peptide information because this method avoids manual data transcription.

Anticipated Results

For a noncleavable pin-peptide synthesis, two control peptides, one of which is reactive with a monoclonal antibody in ELISA and the other serving as a nonbinding peptide control, should show the specific binding expected based on past data. For cleaved peptides, the yield of control peptide should be in the range

expected from the stated pin loading (substitution level), e.g., 1 μmol for GAP and DKP kits or 5 μmol for the MPS kit. Purity of the cleaved controls should be consistent with the results of previous batches and should be of an acceptable standard.

Testing of a systematic set of peptides in a bioassay can give data that is interpretable without recourse to additional controls, because a systematic set of peptides through a protein includes many sequences that are unlikely to be reactive sequences, i.e., they act as internal negative controls. Figure 9.7.7 shows one set of ELISA data from scanning noncleaved peptides with a monoclonal antibody. In screening for T helper cell responsiveness it is critical to include many control cultures, not only controls with no peptide added but also controls

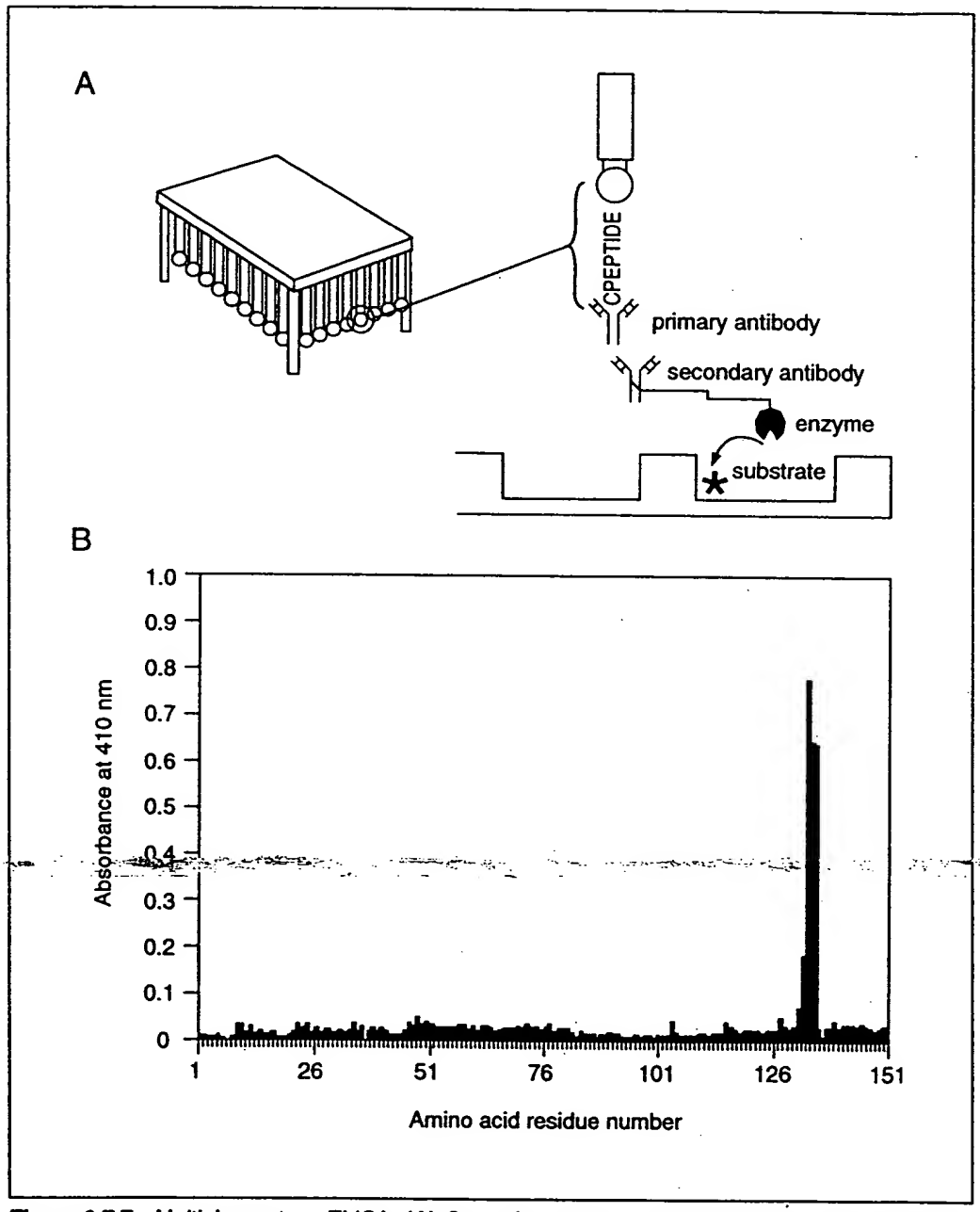

Figure 9.7.7 Multipin capture ELISA. **(A)** Setup for multipin capture ELISA. Pins (gears) with peptides covalently attached are incubated in primary antibody, secondary antibody, and substrate developer in ELISA plates. The absorbance is measured and the resulting absorbance values are graphed versus peptide number, corresponding to the N-terminal residue number of the peptide in the protein sequence. **(B)** Peptide pin capture ELISA results with a monoclonal antibody against pins bearing octamer peptides of gonococcal pilin protein. All the peptides that show high readings contain a significant portion of the epitope. (Diagram courtesy of Dr. Fred Cassels, Walter Reed Army Institute of Research, Washington, D.C.)

with nonstimulatory peptide. Systematic sets of peptides automatically include such controls (Reece et al., 1994).

Time Considerations

If amino acid coupling is carried out at 3 cycles/day, which can fit into a conventional working day, then it will take up to 2 weeks to make a set of 15-mers, as there is extra time required for side chain deprotection and drying down (depending on the peptide format). Although this may seem slow, the fact that hundreds or thousands of peptides can be made simultaneously means that a project requiring large numbers of peptides is completed in a very short time. Indeed, the rate-limiting step may be the time it takes to carry out the assays on the large number of peptides when they become available.

From this perspective, biotinylated peptides produced on glycine acid peptide (GAP), diketopiperazine (DKP), or multiple peptide synthesis (MPS) pins have a great advantage over the noncleavable peptide (NCP) pin-bound peptides, as the latter can only be assayed once a day, whereas hundreds of parallel assays can be carried out on all biotinylated peptides at once. Reading data directly into a computer enables the massive amounts of data to be stored efficiently for later analysis.

Dispensing amino acids can be carried out efficiently by two people, one reading out the position into which the amino acid is to be dispensed and the other doing the actual dispensing. The passive partner (reader) can also act as a cross-checker to ensure no mistakes are made. If a computer-controlled pointing device is used, accuracy is improved and dispensing becomes a one-person operation. For large syntheses (>200 peptides), it is important that the dispensing be fast and accurate so that three couplings can be carried out per day.

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Scanning for T helper epitopes with human PBMC using pools of short synthetic peptides

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Abstract

Major T helper epitopes of medically important antigens can be located by measuring the proliferative responses of human peripheral blood mononuclear cells (PBMC) to pools of short synthetic peptides. The length and endings of the peptides used were shown to be critical for success in identifying Th cell epitopes. Many epitopes would be missed if either long (31mers) or short (less than 12mers) peptides were used. Pools of 14 and 16mers were more efficient than 12mers spanning the same region, however, for a promiscuous Th cell epitope of tetanus toxin (tt 947–967), two of three donors tested did not respond to 18mers or shorter peptides spanning this region. Although peptides with either unblocked or blocked ends were stimulatory, peptides with blocked ends were generally more efficient. The peptide concentration and number of available APC were also found to affect the efficiency of the proliferation assay as a measure of peptide recognition by Th cells.

Two screenings of the entire set of tetanus toxin peptide pools using different samples of PBMC from the same donor identified common major stimulatory regions. Thus, PBMC and peptide pools can be used for the reproducible identification of Th cell epitopes. After immunization with tetanus toxoid (TT), peptide-responsive cells increased in frequency in parallel to the increase in TT responsive cells, indicating that the peptide-responsive cells were primed by TT.

Key words: T helper cell; Epitope; Synthetic peptide; Peripheral blood mononuclear cell; Proliferation; Tetanus toxin

1. Introduction

Mapping of T helper cell epitopes within protein antigens has previously been achieved using the response of Th cell clones to protein fragments generated by various methods, including enzymic or chemical fragmentation (Demotz et al., 1989a,c), gene fragments generated by restriction enzyme digestion (Lamb et al., 1987) or PCR using synthetic oligonucleotide primers (Nakagawa et al., 1991). Synthetic peptides of 15–30 residues (Good et al., 1988; Ho et al., 1990; Brett et al., 1991) and short peptides synthesized using the multipin peptide system (Maeji et al., 1990; Gammon et al., 1990; Brown et al., 1991; Mutch

Abbreviations: TT, tetanus toxoid; tt, tetanus toxin; PBMC, peripheral blood mononuclear cells; β-dkp, β-amino-alanine-diketopiperazine; dkp, diketopiperazine.

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et al., 1991; Suhrbier et al., 1991; Burrows et al., 1992; Rodda et al., 1993; Reece et al., 1993) have also been used.

Work with clonal Th cells has shown that the nature and length of the peptide containing the T cell epitope have important effects on its stimulatory ability. Peptides with blocked N- and C-terminal endings were demonstrated to be just as efficient or more efficient than unblocked peptides for activating T helper cells (Allen et al., 1989; Mutch et al., 1991; Gammon et al., 1991). In contrast, shorter peptides with unblocked endings containing the minimum epitope were shown to be more efficient for cytotoxic T cells (Bednarek et al., 1991).

TT processing and epitope formation by APC has been shown to vary for donors even with the same restriction element (Demotz et al., 1989b; Panina-Bordignon et al., 1989) and inbred mice of the same MHC haplotype (Gammon et al., 1990). This may be due to a requirement for specific protease(s) to generate particular epitopes. The use of shorter peptides, which require little or no processing to be active in Th cell assays (Mellins et al., 1990), avoids the requirement for specific proteases to generate epitopes. Peptides with as few as 8, 9 and 10 amino acids (plus the C-terminal tripeptide β-amino-alanine-diketopiperazine (β-dkp) moiety) have been shown to be stimulatory for Th cell clones (Suhrbier et al., 1991; Brown et al., 1991; Gammon et al., 1991). These lengths are consistent with peptides found bound naturally to class II antigens (Rudensky et al., 1991; Hunt et al., 1992).

Peripheral blood mononuclear cells (PBMC) have been used to map T helper cell epitopes [illegible] using short synthetic peptides (Good et al., 1988; Brett et al., 1991; Russo et al., 1993; Reece et al., 1993). As only the $CD4^+$ subset of T cells is required for in vitro human T cell responses to conventional antigens such as TT (Via et al., 1990), this suggests that proliferation assays involving PBMC are measuring the activation of antigen-specific $CD4^+$ Th cells. PBMC are useful for mapping Th cell epitopes because they represent a repertoire not biased by prior in vitro selection of the best-growing or most frequent clones (Gammon et al., 1990).

A map of the Th cell epitopes of tt using human PBMC and pools of short synthetic peptides has been reported (Reece et al., 1993). The use of PBMC and the peptide pooling strategy has also been applied for mapping Th cell epitopes within other antigens such as influenza type A (two subtypes of HA, and NP) (Rodda et al., 1993); MPB-70 from *Mycobacterium bovis* (unpublished data), Lol pI (Bungy et al., 1993) and HIV antigens; gag and env (Mutch et al., unpublished observations).

This paper reports an assessment of the sensitivity and efficiency of the pooling/decoding method for identifying T helper cell epitopes using PBMC and pools of synthetic peptides. We have assessed the reproducibility of the method and the effect of peptide concentration and length of the peptides on the efficiency of Th cell recognition. Ways of optimizing recognition of Th cell epitopes, such as supplementing PBMC with APC, were examined.

We also addressed the question of whether PBMC responses to short peptides is due to the cross-reactivity of Th cells primed with a different antigen (Good et al., 1992). The specificity of proliferative responses of PBMC to tt peptide epitopes was tested by study of a donor before and after immunization with TT.

2. Materials and methods

To ensure that proliferation assays using PBMC were minimally affected by spontaneous proliferation, factors such as the culture medium, [illegible] incubation time and labeling method were systematically investigated and optimized. All work was performed using the optimized method for the standard PBMC proliferation assay described below, except where specified.

2.1. Medium

'Incomplete' medium consisted of RPMI 1640 (CSL, Melbourne, Australia) supplemented with 2 mM L-glutamine, 5 mM Hepes buffer pH 7.4, and 20 μg/ml gentamicin. 'Complete' medium consisted of 10% (v/v) heat-inactivated human

serum, pooled from screened donations, or 10% (v/v) heat-inactivated autologous serum (obtained from defibrinated blood), added to 'incomplete' medium.

2.2. Antigens

Pin-made peptides were synthesized using the multipin peptide synthesis strategy (Maeji et al., 1990). Peptides had either blocked endings (i.e., an acetylated N-terminus and a C-terminal β-dkp or dkp group) or where noted, unblocked endings (free N- and C-terminal endings). Peptides were cleaved into sterile 0.1 M sodium bicarbonate or 0.1 M Hepes, pH 7.8, in sterile 96-well microtitre trays. The purity of representative peptides was assessed using HPLC and was generally found to be > 80%.

The following bulk peptides were prepared by solid phase peptide synthesis using an Applied Biosystems 430A peptide synthesizer: P388 (H-QYIKANSKFIGITEL-OH, tt 830–844; Panina-Bordignon et al., 1989), P399 (Ac-QEIYMQHT-YPIS-β-dkp, tt 257–268), P442 (H-EQDPSGAT-TKSAMLTNLIIFGPGPVLNKNEV-OH, tt 141–171), P443 (H-SVDDALINSTKIYSYFPSVISKV-NQGAQGIL-OH, tt 581–611), P444 (H-DTQSK-NILMQYIKANSKFIGITELKKLESKI-OH, tt 821–851), P445 (H-IEYNDMFNNFTVSFWLR-VPKVSASHLEQYGT-OH, tt 941–971), P459 (Ac-VRDIIDDFTNESSQKT-NH_2, tt 616–631) and P480 (H-FNNFTVSFWLRVPKVSASHLE-OH, tt 947–967; Panina-Bordignon et al., 1989), P485 (Ac-IVKQGYEGNFIG-OH, tt 652–663), P486 (Ac-STIVPYIGPALN-OH, tt 640–651), P487 (H-EYSYFPSVISK-OH, tt 591–602). Peptides were purified to > 90% and their compositions were confirmed by amino acid analysis.

All peptides were screened for cytotoxic activity by co-culturing with PBMC and 10 μg/ml Con A (Sigma, St. Louis, U.S.A.).

TT was a gift from the Commonwealth Serum Laboratories, Melbourne, Australia.

2.3. Cell preparations

Whole venous blood was drawn from volunteers who had given informed consent to venepuncture. PBMC were isolated from defibrinated or heparinized venous blood using Ficoll-Paque density centrifugation (Pharmacia LKB Biotechnology, Uppsala, Sweden) as described by Bøyum (1968).

2.4. Standard PBMC proliferation assay

Peptide-stimulated proliferation assays using 2×10^5 PBMC per well were performed in 96-well round bottom microtitre plates (Nunc, Roskilde, Denmark). Antigens were added in 20 μl volumes to these microtitre plates followed by 180 μl of cells in complete medium to give a final volume of 200 μl per well. Because PBMC often exhibited a low frequency of T cells specific for individual Th epitopes, all assays were carried out using at least 16 replicates per test group. PBMC were incubated at 37°C in 5%CO_2 in humidified air. After 90 h proliferation was detected by pulsing with 0.25 μCi tritiated (methyl-3H) thymidine (40–60 Ci/mmol, Amersham Australia, Sydney) per well for 6 h. DNA was harvested onto glass fibre filter mats (Skatron, Sterling, VA, USA) and incorporated thymidine was measured in an LKB 1205 Betaplate liquid scintillation counter. All assays included at least 16 wells each of negative controls (20 μl of peptide cleavage buffer) and positive controls (TT at 1.0 or 0.1 Lf/ml in 20 μl cleavage buffer) plus 180 μl cell suspension.

2.5. Statistical methods

Large amounts of peptide-stimulated PBMC data clearly demonstrated that the cpm values for replicate cultures (wells) within each test group were not normally distributed (data not shown). This is a direct consequence of the random distribution of low numbers of peptide-specific Th cells among replicate wells and made it inappropriate to treat proliferation data (cpm) using statistical methods based on the Normal distribution. The Poisson model is a better model for the data (Taswell et al., 1984). To assign the results from individual wells as 'positive' or 'negative', a cutoff value of the mean plus three times the standard deviation of the cpm values for the

normally distributed unstimulated (Cells Alone) group was calculated (Taswell et al., 1984). Poisson statistics were then used to determine whether the difference in the frequency of positive wells between each test group and the Cells Alone group was significant. Where the data are significant at the 0.25% ($p < 0.0025$) or 5% ($p < 0.05$) level, results are reported as the frequency of positive wells in the test groups.

As this method is uncommon for analysis of proliferation tests, a typical set of data comparing this method of analysis with a conventional method using the mean ± SD of the ^{3}H-TdR uptake (cpm) has been reported (Reece et al., 1993). This comparison shows that where significant frequencies are reported, the mean of all replicates in a test group is higher than the mean of the Cells Alone control group, but the SD is also high, so a test based on normally distributed cpm data may not detect a difference between test groups and the Cells Alone controls. In contrast, if individual wells are scored as positive or negative, the statistical test based on a Poisson distribution can be used as a more sensitive and realistic way of looking for test groups significantly different from the Cells Alone control (Reece et al., 1993).

Precursor frequencies were estimated using the single-hit Poisson model (Taswell et al., 1984).

3. Results

3.1. Development of the method for T cell epitope mapping using PBMC

Proliferation of PBMC in response to incubation with sets of short synthetic peptides encompassing entire protein sequences was developed as a method for the detailed mapping of helper T cell determinants (materials and methods section). Dodecapeptides (12mers) with a constant 3-residue C-terminal extension (β-dkp) (Maeji et al., 1990) were employed for this work since previous studies have shown that peptides of this length are suitable for identifying epitopes using Th cell clones (Brown et al., 1991; Suhrbier et al., 1991; Gammon et al., 1991) and fall within the range of peptide lengths found binding naturally to MHC class II (Rudensky et al., 1991). As individual testing of every overlapping 12mer peptide of large proteins such as tt (1304 peptides) was impractical, a pooling/decoding strategy was devised. Using this strategy, the two most commonly recognized regions (Pools 30 and 42) corresponded to two published tt epitopes (Ho et al., 1989; Panina-Bordignon et al., 1989), showing that the pooling method is effective for identifying major Th cell epitopes (Reece et al., 1993).

To investigate whether the PBMC mapping method would give reproducible results with different aliquots of PBMC, one donor was scanned twice at an interval of 2 weeks (Table 1). This assay was performed using the standard proliferation assay (materials and methods section) except that a 138 h incubation period was used rather than 90 h. Results are reported as peptide pools scored positive at the $p < 0.05$ (+) and the $p < 0.0025$ (++) levels.

Table 1 shows that of the ten frequently stimulatory pools identified at the $p < 0.0025$ level in the first assay, seven were also frequently stimulatory ($p < 0.0025$) and two pools significantly stimulatory ($p < 0.05$) in the second assay. Only one pool (pool 12) identified at the $p < 0.0025$ level in the first assay was not stimulatory in the second assay. There were, however, five stimulatory pools (pools 18, 34, 45, 58 and 66) identified in the second assay at the $p < 0.0025$ level that were not identified in the first assay. The difference in the pools identified in the two scans may represent a temporal change in the dominance of epitopes (i.e. clones).

3.2. Comparison of the effectiveness of pools of overlapping 12mer peptides with single 31mer peptides spanning the same regions

Responses of several donors to each of three pools spanning three dominant epitope regions within the tt sequence (Reece et al., 1993) were compared to responses incurred using the single 31mer peptide containing all residues encompassed by that particular pool. A fourth peptide spanning a published 'promiscuous' T helper cell epitope (tt 947–967, Panina-Bordignon et al.,

Table 1
Complete scan of tetanus toxin for Th cell epitopes using two different PBMC samples from donor E [a]

Pool [b] no.	tt sequence spanned by pool	Donor E [c] test 1	Donor E [c] test 2
1	1– 31		
2	21– 51		
3 *	41– 69		
4	59– 89		
5	79– 109		+ [d]
6	99– 129		
7	119– 149		
8	139– 169	+ + [e]	+ +
9	159– 189		+
10	179– 209		+
11	199– 229		
12	219– 249	+ +	
13	239– 269		
14	259– 289		
15	279– 309	+	+
16	299– 329		
17	319– 349		+
18	339– 369		+ +
19	359– 389		
20	379– 409		
21	399– 429		
22	419– 449		
23	439– 469		
24	459– 489		
25	479– 509		
26	499– 529	+ +	+
27	519– 549		
28	539– 569	+ +	+ +
29	559– 589		
30	579– 609	+ +	+ +
31	599– 629		
32	619– 649	+ +	+ +
33	639– 669	+ +	+ +
34	659– 689		+ +
35	679– 709		
36	[illegible]		
37	719– 749		
38	739– 769	+	
39	759– 789		
40	779– 809		
41	799– 829		
42 *	819– 847	+ +	+ +
43	837– 867		
44	857– 887		
45	877– 907		+ +
46	897– 927		
47	917– 947		
48	937– 967		
49	957– 987		
50	977–1007		+
51	997–1027	+	
52	1017–1047		+
53	1037–1067		
54	1057–1087		
55	1077–1107		
56	1097–1127	+	+ +
57	1117–1147		
58	1137–1167		+ +
59	1157–1187	+	+ +
60	1177–1207	+ +	+ +
61	1197–1227		
62	1217–1247		
63	1237–1267		
64	1257–1287	+ +	+
65	1277–1307		
66 *	1297–1315		+ +
Total no. of positive pools [e]		10	14
Cells alone		1/112 [f]	1/112
TT (1.0 Lf/ml)		14/56 [f]	28/56

[a] A standard PBMC proliferation assay, with the exception of a 138 h incubation period, was used.
[b] Each peptide pool consisted of 20 overlapping 12mers (0.3 μM peptide/pool) unless specified by *.
[c] PBMC were from donor E taken at different times.
[d] Peptide pools scored positive at $p < 0.05$ using 16 replicates per test.
[e] Peptide pools scored positive at $p < 0.0025$ using 16 replicates per test.
[f] Number of positive wells over total number of wells used for these controls.

1989), found to be nonstimulatory using pools of 12mer peptides (Reece et al., 1993) was included to see if a single 31mer could detect an epitope where the pooling method had failed to do so.

The concentration of each peptide within the pool was 0.3 μM, whereas individual 31mers were tested over a range of concentrations (8.5,3.4,0.7 and 0.14 μM) to avoid bias against the long peptides due to the possibility of a suboptimal concentration being used. Results are represented as the number of positive wells out of 32 replicates. Only those data significantly different from the Cells Alone control ($p < 0.0025$) are shown (Table 2). To examine whether there was a need to include every possible overlapping 12mer in the pool for it to be stimulatory, pools were also tested as sets containing overlapping pep-

Table 2
Frequency of PBMC responses to pools of 12mer peptides compared to a single 31mer peptide spanning the same sequence as the pool [a]

12mer pool [b]	Donor	Test group						
		Pool [c]			31mer peptide (μM)			
		1 offset	2 offset	3 offset	8.5	3.4	0.7	0.14
Pool 141–171	B	16 [d]	10	13	8	9	8	5
	F	12	9	9	4	7	6	5
	I	24	24	18	21	16	22	21
Pool 581–611	B	3	4	2	– [e]	–	–	–
	F	3	3	–	–	–	–	–
	H	12	3	–	–	–	11	–
	I	8	13	12	–	–	6	3
Pool 821–851	F	8	5	4	–	–	–	–
	H	12	10	10	–	–	–	3
	I	16	20	11	2	13	4	3
Pool 941–971	B	–	–	–	–	–	4	–
	F	–	–	–	–	–	–	–
	H	–	–	–	–	–	–	–

[a] A standard PBMC proliferation assay (materials and methods section) with a 90 h incubation period was employed.
[b] Pool 141–171 corresponds to 31mer P442; pool 581–611 corresponds to 31mer peptide P443; pool 821–851 corresponds to 31mer peptide P444; pool 941–971 corresponds to 31mer peptide P445.
[c] Each pool contained either twenty; ten or seven overlapping 12mer peptides depending on whether the offset was 1, 2 or 3. Individual peptides within each pool were at a concentration of 0.3 μM.
[d] Number of positive wells in test groups significantly different from the Cells Alone control ($p < 0.0025$) using 32 replicates per test.
[e] Indicates not significantly different from Cells Alone ($p > 0.0025$).

tides offset by 1, 2 or 3 residues in their 'start' or N-terminal amino acid.

As expected, peptide pools spanning tt 581–611 and tt 821–851 induced proliferation of PBMC. However, the corresponding 31mer peptides gave little or no stimulation at any peptide concentration tested (Table 2). In contrast, the single peptide P442, spanning tt 141–171, was stimulatory but still tended to give a lower frequency of positives than the corresponding pool. Table 2

Table 3
The effect of peptide length and offset on the frequency of PBMC responses to pools of peptides spanning tt 587–609 [a]

Donor	Peptide offset [b]	Peptide length [c]			
		10mer	12mer	14mer	16mer
B	1	6 [d]	18	23	23
	2	8	14	21	23
	3	–	–	17	14
D	1	–	6	10	14
	2	–	–	9	12
	3	–	5	10	8

[a] A standard PBMC proliferation assay (materials and methods section) with a 90 h incubation period was employed.
[b] Increment in N-terminal residue number of consecutive overlapping peptides in the pool.
[c] Each peptide was tested at a concentration of 1 μM.
[d] No. of positive wells out of 32 replicate wells. Only frequencies of positive wells that were significantly different from the Cells Alone control are shown ($p < 0.0025$). – denotes not significantly different from Cells Alone ($p > 0.0025$).

also shows that as the residue offset increases, the frequency of positive responses tends to decrease.

All donors tested were unresponsive to pool 941–971 and the corresponding 31mer peptide P445, which contains the known T cell epitope tt 947–967 (Panina-Bordignon et al., 1989). As these donors were able to respond to the exact published T cell epitope (Table 4 and data not shown), this suggests that detection of some epitopes is dependent on the choice of 'correct' peptide length (see below).

3.3. The effect of peptide length in scanning with pooled peptides

To investigate the effect of peptide length on the efficiency of detection of Th cell epitopes using PBMC, four sets of overlapping peptides of lengths; 10, 12, 14 and 16 residues (plus the

Table 4
A comparison between the 21mer bulk peptide, P480, tt 947–967 and pools of 12, 15, 18 and 21mer peptides spanning tt 946–968 [a]

Peptide test/ length	Sequence	Donors		
		D	F	I
Pool of 12mers	MFNNFTVSFWLR FNNFTVSFWLRV NNFTVSFWLRVP NFTVSFWLRVPK FTVSFWLRVPKS TVSFWLRVPKSA VSFWLRVPKSAS SFWLRVPKSASH FWLRVPKSASHL WLRVPKSASHLE LRVPKSASHLEQ	+ [b]	– [c]	–
Pool of 15mers	MFNNFTVSFWLRVPK FNNFTVSFWLRVPKV NNFTVSFWLRVPKVS NFTVSFWLRVPKVSA FTVSFWLRVPKVSAS TVSFWLRVPKVSASH VSFWLRVPKVSASHL SFWLRVPKVSASHLE FWLRVPKVSASHLEQ	+	–	–
Pool of 18mers	MFNNFTVSFWLRVPKVSA FNNFTVSFWLRVPKVSAS NNFTVSFWLRVPKVSASH NFTVSFWLRVPKVSASHL FTVSFWLRVPKVSASHLE TVSFWLRVPKVSASHLEQ	+	–	–
Pool of 21mers	MFNNFTVSFWLRVPKVSASHL FNNFTVSFWLRVPKVSASHLE NNFTVSFWLRVPKVSASHLEQ	+	+	+
P480	FNNFTVSFWLRVPKVSASHLE	+	+	+

[a] A standard proliferation assay (materials and methods section) with a 90 h incubation period was employed.
[b] Individual peptides within pools and P480 were tested at 4, 1 and 0.25 μM using 24 replicates per test. Positives (+) were scored if the number of responding wells was significantly different from the Cells Alone control at the $p < 0.0025$ level for any of the concentrations tested.
[c] – denotes not significantly different from the Cells alone control ($p > 0.0025$).

β-dkp tripeptide moiety) spanning the region tt 587–609 (Ho et al., 1990), were synthesized. All peptides of the same length were pooled (1 μM peptide/pool) and tested for their ability to stimulate PBMC from donors B or D. For each peptide length, three pools of peptides were tested, stepping along the sequence with an offset of 1, 2 or 3 residues (Table 3). Results are reported in the same way as in Table 2.

Table 3 shows that as the length of the peptides increases, the frequency of positive responses also increases. In particular, note that 10mer peptides were unable to stimulate PBMC from donor D whereas 14 and 16mer peptides were very effective. As peptide offset increases, the frequency of positive responses decreases, and proliferative responses to pools of shorter peptides (10 and 12mers) offset by 2 or 3 may not be seen. This confirms observations in Table 2 and suggests these pools may not contain the stimulatory sequence, which would be expected if both the length and 'frame' of the epitope within the peptide were important.

For 14mer and 16mer peptides, pools containing peptides offset by 2 were just as effective as those offset by 1, and significant responses were also obtained using 14mer and 16mer peptides offset by 3. These results suggest that a sequence can be successfully scanned with a subset of all possible overlapping peptides (of a given length) spanning the sequence.

3.4. Detailed investigation of the tetanus toxin region tt 947–967 containing a promiscuous epitope

None of the donors tested using the pools of 12mer peptides responded to the region covering promiscuous T cell epitope tt 947–967 (Panina-Bordignon et al., 1989; Reece et al., 1993). Table 2 also shows that the 31mer peptide, P445 (tt 941–971), was not stimulatory for donors B, F and H. In contrast, donors B, D, F, H and I were found to respond to P480, the 21mer peptide corresponding to the published sequence tt 947–967 (Table 4; data not shown for B and H).

To see whether the lack of stimulation by

Table 5
The effect of peptide concentration and unblocked versus blocked endings on the effectiveness of Th cell recognition [a]

Peptide concentration (μM)	Peptide			
	tt 257–268 (donor I)		tt 591–602 (donor E)	
	Blocked endings [b]	Unblocked endings [c]	Blocked endings	Unblocked endings
16	10 [d]	–	7	10
8	–	5	3	18
4	6	–	4	9
2	4	–	3	14
1	–	–	4	10
0.5	–	–	–	8
0.25	–	–	4	5
0.125	–	–	–	–
0.0625	–	–	–	–
0.031	–	–	–	–
0.016	–	–	–	–
0.0078	–	–	–	–

[a] A standard PBMC proliferation assay with a 90 h incubation period was employed.
[b] 12mer peptides with an acetylated amino terminus and a dkp moiety at the carboxy terminus.
[c] 12mer peptides with free carboxy and amino termini.
[d] no. of positive wells out of 32 replicate wells. Only frequencies that were significantly higher than the Cells Alone control ($p < 0.0025$) are shown.
– indicates not significantly different from Cells Alone ($p > 0.0025$).

shorter peptides was due to a need for the stimulatory sequence to consist of more than 12 antigen-homologous residues, four sets of peptides (12, 15, 18 and 21mers) spanning tt 946–968 were tested for stimulation. Peptides of the same length were pooled together (Table 4) and tested in parallel with P480 at three concentrations; 4, 1 and 0.25 μM. Data in Table 4 is reported as positive (+) if the frequency of positive wells was significant ($p < 0.0025$) at any of the concentrations tested.

Of the three donors tested, only donor D responded to pools of all peptide lengths tested (Table 4). In contrast, donors F and I responded to the pool of 21mer peptides and P480, but did not respond to peptides shorter than 21 residues. This indicates that the Th cell epitope for these two donors must be longer than 18 residues for it to be stimulatory.

3.5. The effect of peptide concentration and unblocked versus blocked endings on the effectiveness of T helper cell epitope mapping

Previous reports have shown that peptides with different endings differ in their ability to stimulate Th cell clones (Allen et al., 1989; Mutch et al., 1991; Gammon et al., 1991). The effect of peptide concentration was examined by testing two donors (I and E) against two 12mer peptides with blocked or unblocked endings. Peptides were tested at a range of doubling dilutions from 16 μM to 0.0078 μM. Table 5 shows that the concentration of peptide required to stimulate Th

Table 6
A comparison of the efficiency of individual blocked and unblocked peptides for the identification of a Th cell epitope within the A/Bangkok/1/79 haemagglutinin sequence 425–470 [a]

A/Bangkok [b] start residue	Sequence	Donor [c]					
		Flu1			Flu2		
		Blocked [d] 13mers	Unblocked [e] 13mers	Unblocked 16mers	Blocked 13mers	Unblocked 13mers	Unblocked 16mers
425	LEKYVEDTKIDLW(SYN)	–	–	–	–	–	–
427	KYVEDTKIDLWSY(NAE)	7 [f]	–	–	–	–	–
429	VEDTKIDLWSYNA(ELL)	–	–	14	–	–	–
431	DTKIDLWSYNAEL(LVA)	16	8	13	–	–	–
433	KIDLWSYNAELLV(ALE)	17	10	13	–	–	–
435	DLWSYNAELLVAL(ENQ)	17	12	15	7	–	–
537	WSYNAELLVALEN(QHT)	7	–	–	4	–	6
439	YNAELLVALENQH(TID)	–	–	–	14	–	5
441	AELLVALENQHTI(DLT)	–	–	–	7	–	6
443	LLVALENQHTIDL(TDS)	–	–	–	7	–	–
445	VALENQHTIDLTD(SEM)	–	–	–	–	–	–
447	LENQHTIDLTDSS(MNK)	–	–	–	–	–	–
449	NQHTIDLTDSEMN(KLF)	–	–	–	–	–	–
451	HTIDLTDSEMNKL(FEK)	–	–	–	–	–	–
453	IDLTDSEMNKLFE(KTR)	–	–	–	–	–	–
455	LTDSEMNKLFEKT(RRQ)	–	–	–	–	–	–

[a] A standard PBMC proliferation assay with a 90 h incubation period was employed.
[b] Start residue of peptides spanning A/Bangkok/1/79 haemagglutinin sequence 425–470. Individual peptides were tested at a concentration of 3 μM.
[c] Flu1 and Flu2 refer to two donors known to respond to a Th cell epitope within A/Bangkok/1/79 425–461.
[d] Blocked refers to acetylated peptides with dkp endings.
[e] Unblocked refers to non-acetylated peptides with free acid endings.
[f] no. of positive wells out of 24 replicate wells. Only frequencies that were significantly higher than the Cells Alone control ($p < 0.0025$) are shown. – denotes not significantly different from Cells Alone ($p > 0.0025$).

Table 7
The effect of the addition of APC to P459- and TT-stimulated PBMC on tritiated thymidine incorporation and frequency of positives [a]

Cells used	Cells alone		P459		TT 0.5 Lf/ml
	Mean cpm (SD)	Cutoff [b]	10 μM	1 μM	
PBMC	288 (96)	576	33/48 [991 ± 381] [c]	21/48 [870 ± 332]	48/48 [9651 ± 2726]
PBMC + APC [d]	275 (75)	499	46/48 [1220 ± 590]	45/48 [797 ± 262]	48/48 [8074 ± 1526]

[a] A standard PBMC proliferation assay with a 90 h incubation period was employed.
[b] The cutoff cpm value was calculated as the mean plus 3SD for all the wells in the Cells Alone group ($n = 48$).
[c] The frequency of positive wells out of 48 replicates and mean cpm ± SD of these positively responding wells. All six test groups were significantly different from the Cells Alone ($p < 0.0025$).
[d] APC = 100000 irradiated (3000 Rads) autologous PBMC per well.

cells depends on the donor and peptide sequence tested. For donor I, > 1 μM peptide was required for stimulation whereas for donor E, stimulation was seen around 0.25 μM. Peptides with blocked endings were more effective with the 12mer peptide tt 257–268 (donor I) whereas the unblocked 12mer 591–602 (donor E) was more effective.

The effect of peptide endings was further examined with a set of overlapping 13mer peptides with either unblocked or blocked endings, and a set of 16mer peptides with unblocked endings, spanning a stimulatory region of the influenza strain A/Bangkok/1/79 hemagglutinin sequence (Benstead et al., in preparation). The individual peptides were tested at 3 μM against two donors and results are presented as the number of positive wells out of 24 replicates for test groups differing from the Cells Alone controls ($p < 0.0025$) (Table 6).

For both donors, the frequency of positive responses to acetylated 13mer peptides with a β-dkp ending was higher than for peptides with free endings (Table 6). However, the 16mer peptides with free endings were of similar effectiveness to 13mer peptides with blocked endings. This indicates that longer peptides are more effective, so if peptides with free endings are used it would be advisable to use peptides of about 16 residues length.

3.6. The effect of the addition of APC on the frequency of positive responses of PBMC to peptide

Orosz et al. (1987) showed that for optimal proliferation when using low numbers of PBMC/well, supplementation with additional APC was necessary. Consequently, we investigated the effect of adding APC (irradiated autologous PBMC) to 2×10^5 PBMC/well to see if the availability of APC was a limiting factor.

PBMC from donor I, known to have a low Th precursor frequency to the tt peptide P459 (data

Table 8
Estimations of precursor frequencies/100000 PBMC, before and after immunization with TT: donor CM16 [a]

Test group	Peptide (1 μM)								TT (Lf/ml)		NP (μg/ml)
	P388	P399	P442	P459	P480	P485	P486	P487	1.0	0.1	110
Before [b]	0.2 [d]	0.9 *[c]	1.0 *	0.1	0.2	0.3	1.8 *	0.39	9.49 *	1.97 *	9.08 *
After [c]	6.3 *	13.1 *	18.5 *	0.8	1.3	6.9 *	24.6 *	1.74	> 77.43 *	76.58 *	9.81 *

[a] A standard PBMC proliferation assay with a 90h incubation period was employed.
[b] PBMC isolated just prior to immunization with TT.
[c] PBMC isolated 3 weeks after TT immunization.
[d] Precursor frequency estimations of the no. of peptide and antigen specific Th cells/100000 PBMC.
[c] *denotes significantly different from Cells Alone ($p < 0.0025$). More replicates were used in the Cells Alone group in the before group, giving these precursor frequency estimations a higher precision.

not shown), were tested with and without the addition of 1×10^5 irradiated PBMC (3000 Rads) (Table 7). The addition of APC increased the frequency of positive responses to tetanus toxin epitope P459 (tt 616–631) at 10 μM and 1 μM without significantly altering the magnitude of thymidine incorporation (Table 7). This indicates that the number of APC is limiting even when using 2×10^5 PBMC per well, and suggests that to detect all specific Th cells, extra APC need to be added to PBMC.

3.7. Are proliferative responses to tt peptides a result of Th cells responding to cross reactive antigens?

To confirm that PBMC responses to tt-homologous peptides are due to Th cells primed by TT rather than by cross reactive antigens, we examined proliferative responses of PBMC to a series of common tt Th cell epitopes before and after immunization of a volunteer with a TT booster (Table 8).

Significant increases in the precursor frequency estimations of Th cells specific for peptides P388, P399, P442, P485, P486 and TT were found 3 weeks after the TT immunization. In contrast, the frequencies of Th cells to two control antigens, influenza nucleoprotein (NP) and PPD (data not shown) did not alter after the TT immunization.

4. Discussion

We sought to combine the use of short synthetic peptides, which require little or no processing to be active in T helper cell assays (Mellins et al., 1990), with the use of PBMC as a source of polyclonal T cells. Although the mapping of Th cell epitopes using PBMC provides a repertoire not biased by the in vitro selection of the best-growing or most frequent clones (Gammon et al., 1990), working with PBMC poses difficulties not found when working with Th cell clones.

One major difficulty working with PBMC is the occurrence of sporadic (non-antigen-specific) proliferation in unstimulated PBMC cultures. The occurrence of 'false' positives is increased when culture conditions are not optimized, so factors such as media components must be screened to ensure the lowest background stimulation, while still supporting strong antigen-driven proliferation. A second difficulty arises due to the relatively low frequencies of Th cell precursors specific for a particular epitope. A large number of cells must therefore be used to ensure that significant numbers of peptide-specific Th cells are present. These can be distributed into a large number of replicate wells per test group to permit a statistical test of the difference between the control (Cells Alone) and each test group to be made. Based on theoretical considerations and our observations, we determined that conventional methods of data analysis (S.I., mean $\pm$ SD, net cpm etc.) were inappropriate, so an algorithm was developed which calculates a cutoff cpm value to enable those wells exhibiting significant proliferative responses to be objectively scored. A statistical test using Poisson statistics then differentiates positive tests groups from negative test groups and the Cells Alone controls (Geysen et al., in preparation).

A further restriction when using short synthetic peptides is the large number of peptides required to scan a whole protein sequence. This restriction can be overcome by synthesizing peptides using multipin peptide synthesis systems (Bray et al., 1990; Maeji et al., 1990). Testing overlapping peptides as pools reduces the limitation created by the limited availability of PBMC. The peptide pooling strategy was found to be an effective and simple method for identifying immunodominant Th regions within a protein (Bungy et al., 1993; Rodda et al., 1993; Reece et al., 1993).

To assess the effectiveness of the peptide pooling strategy compared to a conventional method using longer synthetic peptides (Good et al., 1988; Ho et al., 1990; Brett et al., 1991), pools of 12mer peptides were compared with 31mers spanning the same sequence. These results showed that the pools of short peptides were more efficient than longer peptides (Table 2). There may be a block in the recognition of longer synthetic peptides by helper T cells due to poor uptake from the medium or a requirement for specific protease(s)

to generate particular epitopes. Antigen processing and epitope formation by APC has been shown to vary even for donors with the same restriction element (Panina-Bordignon et al., 1989) and inbred mice of identical MHC haplotype (Gammon et al., 1990).

The most efficient length of peptide for specific stimulation of PBMC is unknown. Factors such as uptake by MHC class II, and whether or not processing of the peptide is necessary, affect the efficiency of epitope detection using polyclonal T cells (PBMC). Although pools of 12mer peptides were shown to be more effective than 31mers, we found that slightly longer peptides (up to 16 residues) were even more efficient for identifying Th cell epitopes (Table 3). Peptides in the 13–18 residue range have been found by extraction from purified class II molecules (Rudensky et al., 1991; Hunt et al., 1992) which suggests that synthetic peptides similar in length to the native peptides are the most efficient for detecting T cell epitopes.

In one instance short peptides were not successful in identifying a major Th cell epitope. Donors did not respond to pools of 12mer peptides spanning the promiscuous Th cell epitope tt 947–967 (Panina-Bordignon et al., 1989) even though they responded to a peptide corresponding to the published 21mer sequence (Reece et al., 1993). Further screening of 12, 15, 18 and 21mer peptide pools showed that of the three donors tested, only one responded to all peptide lengths while the other two responded only to 21mer peptides (Table 4). Thus, for some donor/epitope combinations, the peptide must be greater than 18 residues long to be stimulatory.

Overall these results show that some Th cell epitopes may be missed if peptides are too long (31 residues) or too short (12 residues). Therefore a peptide length between 16 and 21 residues is probably suitable for the identification of the majority of Th cell epitopes. Pools containing 16mer peptides offset by two residues can be just as efficient as pools of peptides offset by 1 (Tables 2 and 3), halving the number of peptides otherwise required for complete scanning.

Because short peptides require little or no processing to be active in Th cell assays (Mellins et al., 1990), some peptide responses may occur as a result of activating Th cells primed with a cross-reactive antigen (Good et al., 1992). To investigate if responses to tt peptides were due to priming with TT, we examined changes in the frequencies of Th cells to TT, or to tt-homologous peptides, as a result of immunization with TT (Table 8). The frequencies of Th cells to five tt peptide epitopes increased in parallel with increased frequencies to TT, indicating that PBMC responses to tt peptides are not due to Th cells generated to fortuitously cross reactive antigens. A specificity test (Hensen and Elferink, 1984) of PBMC responses also showed specificity of peptide-reactive PBMC for TT, and vice versa (data not shown).

We found that peptides with blocked endings were as efficient or more efficient for activating Th cells than unblocked peptides (Tables 5 and 6). This confirmed previous observations using Th cell clones (Allen et al., 1989; Mutch et al., 1991; Gammon et al., 1991) and may be due to the resistance of peptides with blocked endings to proteolytic action. These results contrast with cytotoxic T cells where shorter peptides with unblocked endings containing the minimum epitope were found to be more efficient (Bednarek et al., 1991).

The concentration of peptide required to stimulate Th cells depended on the peptide and donor tested. For one donor-peptide system, >2 μM peptide was required for stimulation whereas another peptide-donor system required only 0.25 μM peptide. This information implies that some Th cell epitopes may not have been detected using 0.3 μM of each peptide in peptide pools (Reece et al., 1993), even though the effective concentration can be two or three times this level due to epitope sharing between overlapping peptides. Collawn et al. (1989) also showed that although most Th clones respond to 0.3 μM peptide, the optimum concentration can be higher or lower.

Although the frequencies of Th cells for many other antigens are lower than for TT (Van Oers et al., 1987), we have found this method for epitope scanning with PBMC can still be applied

(Bungy et al., 1993). In summary, the distinctive features of the method as described include (i) the medium, especially the use of autologous serum; (ii) the incubation conditions, especially the use of round-bottom wells and a short incubation (90 h); (iii) data analysis which recognizes the spontaneous occurrence of positives in control and test groups; (iv) the use of large numbers of replicates to allow proper statistical evaluation of data; (v) pooling of short peptides; (vi) the choice of peptide length in the 14–21 residue range, a size not requiring further processing for presentation; and (vii) the use of shorter peptides which are active when their N- and C-terminal ends are blocked.

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